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CYTOLOGICAL OBSERVATIONS ON HYPOXIS:

III. EMBRYO-SAC DEVELOPMENT IN *HYPOXIS ROOPERII* AND *H. FILIFORMIS*

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Abstract

Embryo-sac development was studied in *Hypoxis filiformis* ($n = 7$) and *H. rooperii* ($2n = \pm 96$). In both cases there is degeneration of the megaspore (or megaspore mother cell) and it looked as if the embryo-sac is produced from nucellar cells. *Hypoxis rooperii* ovules very commonly have two embryo-sacs. Suggestions were made for future research.

Data presented in the first two papers (Wilsenach, and Wilsenach & Papenfus) led to a hypothesis that at least some of the *Hypoxis* "species" are apomictic. Of the "species" studied *Hypoxis rooperii* had the most convincing apomictic features: the plants have a high somatic chromosome number (96), meiosis is abnormal, pollen grains are formed which contain a variable number of chromosomes (43 to 58) and the populations show morphological variation. This "species" has therefore been selected for a study of embryo-sac development in order to test the assumption that it is apomictic.

Hypoxis filiformis represents the other extreme—it has a low chromosome number ($n = 7$) and this number is present in all the generative nuclei in the pollen tubes. The populations also show very little morphological variation. This "species" was selected for embryo-sac development study because it appeared to be the most likely one to have normal sexual reproduction.

Methods

Inflorescences in various stages of development were collected from plants growing in their natural habitat, and were fixed in Randolf's Modified Navashin Fluid as quoted by Johansen (1940). The material was dehydrated in normal butyl-ethyl alcohol mixtures, wax-embedded and the section thickness was 15 to 20 micron. The sections were stained in Fast Green and Safranin.

THE EMBRYO-SAC DEVELOPMENT OF HYPOXIS FILIFORMIS

(i) *The integuments and type of ovule.*

This is not shown in the diagrams, but the ovule is anatropous, and the integuments are evident from the earliest stages recorded here, e.g. in the ovule illustrated in Fig. 1A, the inner integument already covered two-thirds of the nucellus.

(ii) *Megasporogenesis.*

The structure of the megaspore-mother-cell (m.m.c.) was not recorded, the earliest stage recorded was the tetrad of megaspores as seen in Fig. 1A. From this it is evident that the tetrad is T-shaped, that it is surrounded by big nucellar cells, and it regularly "sits" on a basal cell (b.c.). A sub-epidermal archesporial cell obviously gives rise to the megaspores directly, since there are no cells between the tetrad and the nucellar epidermis (n.e.). In Fig. 1A one can see that a periclinal division took place in one cell of the nucellar epidermis.

(iii) *The fate of the megaspores.*

The three micropyle-end megaspores show signs of degeneration at a very early stage (see Figs. 1A, 1B and 1C). The fate of the fourth megaspore is not at all easy to determine. In Fig. 1C this megaspore also shows signs of degeneration, which is evident by a change of the staining (as soon as cells degenerate the cytoplasts stain red compared with the typically green cytoplast after safranin—fast green staining). In Fig. 1D the interpretation is as follows: All four megaspores have degenerated, the two micropyle-end megaspores can be recognised by the position below the nucellar epidermis and the other two have become separated from them by the nucellar cells labelled n.c. The position of the chalazal end megaspore relative to the basal cell (b.c. in all the figures) is considered to be a helpful criterion in the identification of this cell.

(iv) *The origin of the embryo-sac.*

As soon as degeneration of the megaspores takes place some of the elongated nucellar cells (e.n.) begin to enlarge considerably, and this must result in distortion within the ovule. In Fig. 1E a two-nucleated embryo-sac can be seen, with degenerated cells laterally next to it, but no degenerated cells between it and the nucellar epidermis. The most plausible explanation is that some of the cells labelled "d.c." in Fig. 1E must represent the four degenerated megaspores and that this embryo-sac was produced by an enlarged nucellar cell, which will explain why no degenerated cells can be seen between it and the nucellar epidermis.

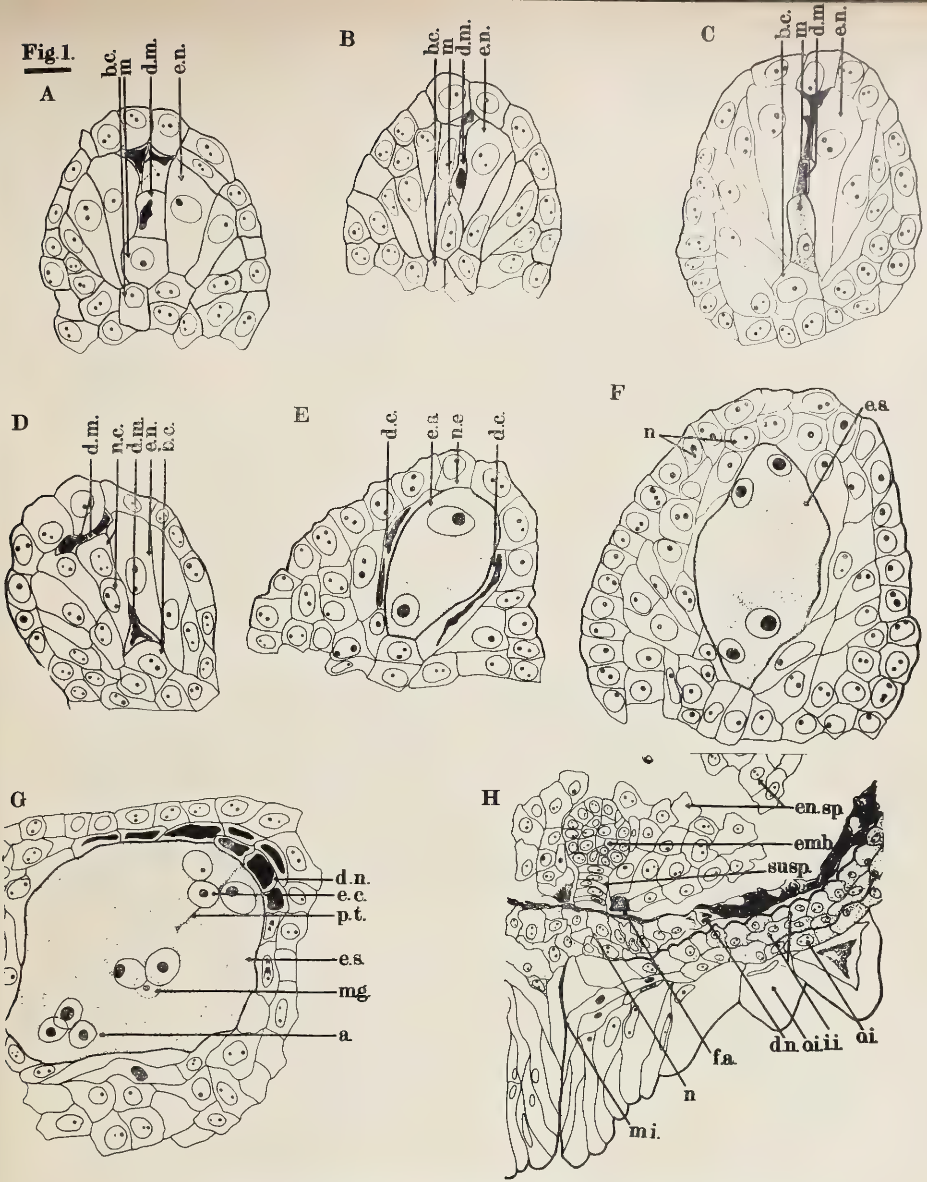

Fig. 1. Embryo-sac development in *Hypoxis filiformis*.

A. Young ovule showing T-shaped tetrads, of which the three micropylar megaspores have degenerated (d.m.).
B. Similar ovule.
C. Slightly older ovule, chalazal megaspore (m.) now also showing signs of degeneration.
D. Ovule with all 4 megaspores in degenerated condition (d.m.).
E. Ovule with bi-nucleated embryo-sac, with degenerated cells laterally (d.c.). Suggested origin of embryo-sac is from an enlarged nucellar cell.
F. Four-nucleated embryo-sac (e.s.), nucellar epidermis double (n.) as result of periclinal divisions of original nucellar epidermis.
G. Eight-nucleated embryo-sac. Male gamete (m.g.) can be seen next to polar nuclei, and signs of male gamete entry can be seen (p.t.).
H. Ovule with embryo (emb.) suspended in endosperm (en. sp.).

a. antipodals; b.c. basal cell; d.m. degenerating cells; d.n. degenerating nucellar cells; e.c. egg cell; e.n. enlarging nucellar cell; e.s. embryo sac; emb. embryo; en. sp. endosperm; f.a. filiform apparatus; i.i. inner integument; m. megaspore; m.g. male gamete; m.m.c. megaspore mother cell; n. nucellus; n.e. nucellar epidermis; o.i. outer integument; p.t. signs of male gamete entry; susp. suspensor.

(v) *The four-nucleated embryo-sac* (Fig. 1F).

Two nuclei are arranged at the micropyle end, two at the other end and a big vacuole is present between them. The periclinal divisions which started earlier (see e.g. Fig. 1A) have continued, with the result that the nucellar epidermis has become two layered (labelled "n" in Fig. 1F).

(vi) *The eight-nucleated embryo-sac.*

As the embryo-sac enlarges some of the nucellus degenerates ("d.n." in Fig. 1G). The eight nuclei are arranged in the typical 3-2-3 arrangement, the nuclei are all small but no filiform apparatus can yet be seen in the synergids. A faint line, labelled "p.t." represents signs of the entry of the male gamete (m.g.) which can now be seen next to the two polar nuclei.

(vii) *The embryo, endosperm and the integuments.*

Stenar (1925) described helobial endosperm development in *Hypoxis.* Our observations are incomplete, but at an early stage two masses of cytoplasm with free nuclei can be seen—one at the micropylar end, one mass at the chalazal end. Subsequent wall formation results in a mass of cellular endosperm at the micropylar end and a disc of cellular endosperm at the chalazal end. These observations are what one would expect to find in helobial endosperm development.

The embryo lies embedded in the endosperm and is attached to a small nucellar cap by a short suspensor.

Markotter (1936) found that the seeds of related genera require a prolonged after-ripening period—it is possible that the embryo then becomes more differentiated. This will explain the failures we have met with in our efforts to germinate freshly collected seed.

The testa of the mature seed is formed by the integuments, both of which remain largely two-layered. The epidermis becomes so thickened that the lumina of the cells are practically obliterated.

To summarise: Megasporogenesis apparently occurs normally, it is followed by the early degeneration of the three micropyle-end megaspores, but the remaining megaspore soon also shows signs of degeneration. It is suggested that a nucellar cell then produces the embryo-sac which becomes the typical 8-nucleated structure. Pollen tube entry has been recorded, fertilization of polar nuclei apparently takes place but the embryo, it is suggested, develops from the egg cell without fertilization.

HYPOXIS ROOPERII

(i) *The megaspore-mother-cell and its fate.*

At very early stages it is easy to recognise the megaspore-mother-cell (m.m.c.) as a much enlarged cell in the nucellus. It is surrounded by large elongated

FIG. 2.

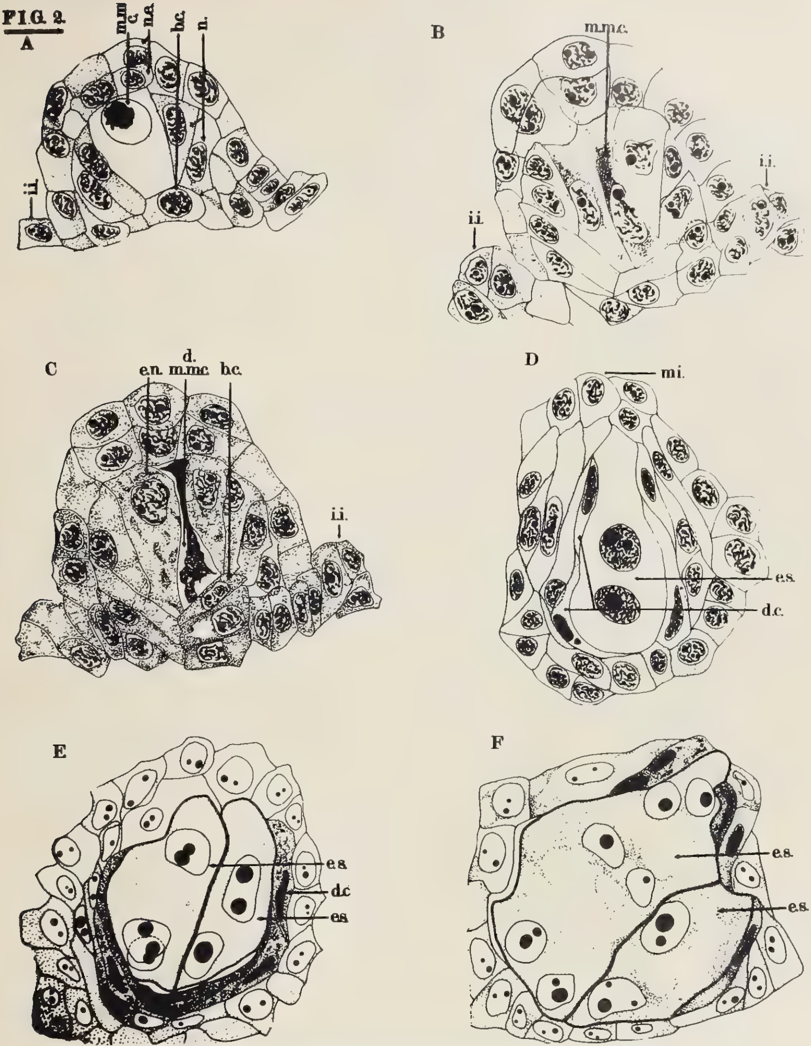

FIG. 2. Stages in embryo-sac development in *Hypoxis rooperii*.

A. Young ovule with megaspore mother cell (m.m.c.), showing beginning of the inner integument (i.i.) and doubling of the nucellar epidermis (n.e.).
B. First signs of degeneration of the m.m.c.
C. Degenerated m.m.c., which is being squashed by an enlarging nucellar cell (e.n.).
D. Two-nucleated embryo-sac, suggested origin is from an enlarged nucellar cell. Degenerated cell (d.c.) must represent the displaced degenerated m.m.c.
E. Twin embryo-sacs, a very common occurrence.
F. Later stage of two embryo-sacs, one shows 6 nuclei and the other 2.

nucellar cells, and there is a layer of cells between the m.m.c. and the nucellar epidermis (Fig. 2A).

There seems to be some variation in the immediate development of the m.m.c. In one case an anaphase of meiosis I could be observed but in most cases there was very early degeneration of the m.m.c. as is illustrated in Fig. 2C. Fig. 2B represents an earlier stage where the m.m.c. becomes pink, which is a sign of early degeneration.

The identification of the degenerated cell as the m.m.c. is possible at early stages. This explains why meiosis was observed in only one slide and why tetrads could never be seen.

As soon as the m.m.c. degenerates an adjacent nucellar cell enlarges (Figs. 2B and 2C) and encroaches upon it so that it will become displaced. In Fig. 2D such displaced degenerated cells can be seen (d.c.).

(ii) *The origin of the embryo-sac.*

The chromosome number of the cells is so high that it was impossible to determine the ploidy of the different cells, which makes interpretation of subsequent stages extremely difficult.

In Fig. 2D a two-nucleated embryo-sac can be seen, and it is interpreted as being derived from the enlarged nucellar cell. At least one of the degenerated cells should be the remains of the m.m.c.

(iii) *Twin embryo-sacs.*

Twin-embryo-sacs are subsequently seen very commonly (Figs. 2E and 2F), of which one is invariably bigger than the other. In Fig. 2F one bi-nucleated and one six-nucleated embryo-sac can be seen, in Fig. 2E one is bi-nucleated and the other one possesses at least three nuclei. The origin of the second embryo-sac could not be determined, and two interpretations are possible:

(*a*) They are both formed from the nucellar cells.

(*b*) One has an aposporic origin, the other from the megaspore.

We favour the first interpretation because the rule at early stages is degeneration of the m.m.c., whereas the rule at later stages is twin embryo-sacs; and furthermore it must be remembered that tetrads were never observed. Even if this interpretation is not correct, at least one embryo-sac must have an aposporic origin.

Similar twin embryo-sacs were also observed by Joubert (unpublished) in *Hypoxis costata.*

DISCUSSION

Results in the previous two papers (Wilsenach, Wilsenach & Papenfus) have indicated the probability that at least some *Hypoxis* populations are apomictic, which led to the studies which are reported in the last paper on embryo-sac development. It must be realised that it is extremely difficult to demonstrate that a megaspore-mother-cell (m.m.c.) (or the megaspore) degenerates and that it is replaced by a nucellar cell, because once the cell is in a degenerated condition it is almost impossible to establish whether it represents a megaspore or a nucellar cell.

Stenar (1925) reported on embryo-sac development of two *Hypoxis* species viz. *H. decumbens* L. and *H. villosa* L. (These have since been combined, and are now considered to be synonyms). She made some observations which are confirmed in the present study e.g. the m.m.c. originates from an archesporial cell which lies below the nucellar epidermis, the megaspore tetrad is T-shaped, the nucellar epidermis divides periclinally, the ovule is anatropous and two integuments are present. Unfortunately her drawings of the embryo-sacs do not include the adjoining nucellar tissue—an aspect that would have been of great interest to us. Stenar described normal monosporic eight-nucleatedembryo-sac development, but she does record the occurrence of two two-nucleated embryo-sacs per ovule as exceptions. She suggests three explanations: (*a*) they are formed by two embryo-sac mother cells (presumably meaning two functional megaspores); (*b*) they were formed because no cell wall was produced after the second meiotic division; (*c*) they were formed by two megaspores from one tetrad.

In the present study such double embryo-sacs occurred commonly in *H. rooperii*, and the phenomenon was accompanied by degeneration of the megaspore mother cell, so that their origin is from nucellar cells. This possibility was not mentioned by Stenar, and it is very interesting to note that she could not observe any degenerating megaspores next to the two young embryo-sacs—it could be that the m.m.c. degenerated at an early stage, or that the archesporial cell formed one of the embryo-sacs without undergoing the reduction division, the other embryo-sac being formed by a nucellar cell. Such events will very easily be overlooked unless one is on the look-out for these occurrences as a result of earlier data which strongly suggest the presence of apomixis.

It has come as a surprise to us that even *H. filiformis* (n = 7) shows definite signs of apomixis. If this can be confirmed apomixis could quite well occur in nearly all our South African species.

Where does all this leave us?

Firstly, it must be pointed out that apomixis has not been confirmed without doubt, but everything points to this: The plants are very polymorphic, there is great variation in chromosome number, meiosis of pollen mother cells shows many abnormalities in the high chromosome number species, pollen grains possess different numbers of chromosomes in these plants, megaspores (or megaspore mother cells) degenerate and twin embryo-sacs are common. More research is necessary to confirm the idea that these plants are apomictic and this will involve: (*a*) more detailed studies of embryo-sac development of different so-called "species"; (*b*) more studies of somatic chromosome numbers, and here some statistical treatment of populations is necessary; (*c*) studies of meiotic behaviour of more populations; (*d*) studies involving genetical experiments. These could quite easily establish apomixis, but one is confronted with the problem that the seeds need a long after-ripening period, making such experiments very time consuming. The most obvious first step is to record the variation in the offspring of open pollinated specimens.

In the meantime a revision of the genus should not be attempted and taxonomists should refrain from the temptation of describing new species. Dobzhansky (1941), Babcock and Stebbins (1938) and Stebbins (1951) have put it quite clearly: The species concept as applied to sexual organisms is not reality in agamic complexes. As Stebbins pointed out: "Disputes between systematists not fully conscious of the biological situation in these groups are particularly academic and futile." Babcock and Stebbins (1938) suggested that the clearest concept of interrelations and phylogeny of the agamic complex is obtained by drawing arbitrary species boundaries which are related to the original boundaries of the sexual species ancestral to the complex. Unfortunately we cannot even suggest the ancestral basic chromosome number, and it will involve many years of intensive study to reveal the sexual ancestors. In the meantime *Hypoxis* will remain a "headache to the systematist". What an unhappy way of ending a series of three papers!

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